Knockdown resistance mutations contributing to pyrethroid resistance in *Aedes aegypti* population, Saudi Arabia

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Abstract

**Background:** Dengue is endemic in Saudi Arabia especially at Jeddah, Makkah, Asir, and Jazan areas where pyrethroids are widely used to control the vector, *Aedes aegypti*. Resistance of *Ae. aegypti* to pyrethroid insecticides has been reported from most of these areas.

**Aims:** The present study was carried out in Jazan region in south-west Saudi Arabia to explore the resistance status of *Ae. aegypti* to pyrethroids and the consequent underlying mechanisms.

**Methods:** Three pyrethroids (permethrin, lambda-cyhalothrin, and cyfluthrin) were used to investigate the resistance status of *Ae. aegypti* adults following WHO standard methods: PCR and sequencing techniques were used to detect the S989P, V1016G and F1534C kdr mutations.

**Results:** *Ae. aegypti* populations were susceptible to cyfluthrin and having a possibility of resistance to permethrin while resistant to lambda-cyhalothrin. Three potential *kdr* mutations were detected for the first time in *Ae. aegypti* population, F1534C, V106G, and S989P. It was found that F1534C often co-exists with V1016G and this haplotype was strongly associated with permethrin and lambda-cyhalothrin resistance. On the other hand, S989P mutation was detected as RR in 18.8% with a low-frequency rate (R) of 18.8%, and in 55.5% as R with 58.3% frequency rate in permethrin and lambda-cyhalothrin-resistant female mosquitoes, respectively.

**Conclusions:** Early detection of resistance alleles considered the golden and essential tool for the successful implementation of insecticide resistance management strategies by providing early warning of insect resistance.

Keywords: dengue, pyrethroids, *Aedes aegypti*, resistance, mutations

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Introduction

The *Aedes aegypti* mosquito is by far the most competent vector of many arboviral diseases, including dengue, yellow fever, chikungunya, Zika and West Nile. Dengue fever and dengue haemorrhagic fever have consequently spread through more than 100 countries in tropical and subtropical zones, resulting in more than half of the world population being at risk (1). Using insecticides to control vector-borne diseases is still the main intervention although efforts to introduce licensed vaccines have progressed greatly.

Dengue is endemic in Saudi Arabia, especially in the Jeddah, Mecca, Asir and Jazan areas. Around 12 131 confirmed cases were reported from Jeddah and Mecca between 2013 and 2015 (2). Likewise, 1790 confirmed cases were reported from Jazan region between 2005 and 2016, with a severe outbreak in 2016 (555 cases). The number of confirmed cases from Jazan region in 2017 was 320 (3).

Knockdown resistance (*kdr*) is a mechanism that describes cases of resistance to pyrethroid as a result of target site insensitivity due to point mutations in the insect voltage-gated sodium channel (VGSC) regulatory protein which block pyrethroid and DDT action (genetic makeup) (4). Several *kdr* mutations have been reported in *Ae. aegypti* populations worldwide; these include G923V, L982W, I1011M/V,S989P, V1016G/I, F1534C and D1763Y (5).

The majority of resistance-associated mutations are found in segment 6 of domain II (IIS6) and domain III (IIIS6) of the sodium channel gene. For instance, valine to glycine in domain II (V1016G) is associated with resistance to type I and type II pyrethroids, such as permethrin and deltamethrin (6), while phenylalanine to cysteine substitution at position 1534 within domain III (F1534C) is associated with resistance to type I pyrethroids (7). On the other hand, serine to proline (S989P) in domain II in VGSC has also been associated with pyrethroid resistance (8) and valine to isoleucine transversion in domain II (V1016I) contributed to *Ae. Aegypti* pyrethroids resistance in Latin America (9). However, S989P has not been found alone (10).

The *kdr* mutations in *Ae. aegypti* have been reported from Singapore (11), China (12,13) and Greece (13), and 1534Leu and 1534Ser have been found in the United States of America (14).

Few studies have been reported on the resistance status of *Ae. aegypti* to insecticides in Saudi Arabia (15–17). Furthermore, studies on mechanisms of resistance to pyrethroids in *Ae. aegypti* populations from Saudi Arabia are lacking. Only one has been conducted in Jeddah and Mecca,
in the western region of Saudi Arabia (2). This study reported 2 mutations (V1016G and S989P) in *Ae. aegypti*, which were shown to be responsible for the resistance of permethrin and deltamethrin.

To the best of our knowledge, no studies have been carried out to investigate the resistance mechanisms of *Ae. aegypti* to pyrethroid insecticides in Jazan region. The aim of this study is therefore to explore the resistance status of *Ae. aegypti* to pyrethroids and the underlying mechanisms.

**Methodology**

**Study area**

Jazan region is situated in the subtropical zone, south-western Saudi Arabia, lies between 16° 12’, and 18° 25’ north. It is surrounded by the Red Sea (260 km) from the west, by Yemen (120 km) from the south and east and by Asir region from the north, with a total area of about 22 000 km² and a population of 1.3 million (18).

**Adult bioassay**

This study was carried out in 2017. Larvae of *Aedes aegypti* were collected from Gizan City and were left to develop till adult under laboratory conditions, 25 ± 2 °C and 75% relative humidity with a constant photoperiod; 12 h light, 12 h dark.

About 100 sugar-fed, 3–5-day-old *Ae. aegypti* female mosquitoes were used for each of permethrin, lambda-cyhalothrin and cyfluthrin for bioassay testing. A batch of 25 adults was introduced into a holding tube before being exposed to insecticide-impregnated papers. Equal numbers of control tests were also carried out by exposing mosquitoes to insecticides–free papers. The experiment was replicated 4 times. After a period of exposure of 60 min under laboratory conditions (25 ±2 °C and 75% relative humidity with a constant photoperiod; 12 h light, 12 h dark), all mosquitoes were transferred to new tubes, provided with 10% sugar solution and held for 24 hours recovery period (19). Mortality was recorded and resistance status was determined as per WHO criteria, i.e. a population is considered susceptible if the mortality rate is (98–100%), having a possibility of resistance 90–97% and resistant < 90% (20).

**Insecticides**

The insecticides used in this study for the adult bioassay tests were the diagnostic dosages as specified in the WHO standard methods. The insecticides were obtained from the WHO Collaborating Centre in Malaysia (Vector Control Research Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang). Adults were tested against 3 pyrethroids (permethrin 0.75%, lambda-cyhalothrin 0.05% and cyfluthrin 0.15%).
Mosquito samples

After adult bioassay, we used the resistant and susceptible mosquitoes against the 3 pyrethroids to detect kdr mutations.

We used 16 permethrin-resistant mosquitoes, 18 lambda-cyhalothrin-resistant mosquitoes, 15 permethrin-susceptible mosquitoes, 20 lambda-cyhalothrin-susceptible mosquitoes and 20 cyfluthrin-susceptible mosquitoes as samples to detect the S989P, V1016G and F1534C kdr mutations.

DNA extraction

After removing the mosquito abdomen, samples were homogenized individually using a mortar and pestle (mini borosilicate glass chamber length 60 mm pestle, diameter 9.0 mm 3.0 mL, Fisherbrand) in 100 μL of Minimum Essential Media (EuroClone, UK).

We extracted DNA from the stored homogenate using RealLine DNA-Extraction 2 kit (BIORON Diagnostics, Ludwigshafen, Germany) following the manufacturer’s recommendations. The extracted DNA was stored at –86 °C till the next procedure.

Detection of S989P, V1016G and F1534C kdr mutations

To detect S989P, V1016G and F1534C mutations, primers (Table 1) and AS-PCR were used according to the procedure described by Li et al. (21).

We carried out AS-PCR in 2 mix reactions for each sample to detect 1 mutation. The 2 mix reactions of 25 μL contained the same reagents [12.5 μl GoTag®G2 green master mix ready-to-use (Promega, Madison, Wisconsin), 3 μl DNA sample] except that 1 mix contained 25 μM of each of the mutant-specific primers and the other contained susceptible-specific primers.

The thermal cycling incubations were as follow: 94 °C initial denaturation for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60 °C (for V1016G and F1534C) or 62 °C (S989P) for 30 s and extension at 72 °C for 60 s and a final extension at 72 °C for 5 min. The PCR products were analysed using gel electrophoresis (1.5 agarose in Tris-Acetate EDTA buffer) staining with ethidium bromide. The visualization was carried out using Gel Doc XR Imaging System (Bio-Rad, Hercules, CA; USA).

Sequencing and bioinformatics analysis

Purification and standard sequencing for RT- PCR products were performed by Macrogen, Korea. Sequencing reactions were performed in a MJ Research PTC-225 Peltier Thermal Cycler using a ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits with Applied Biosystems AmpliTaq DNA polymerase (FS enzyme) (Thermo Fisher Scientific, Waltham, Massachusetts)
following the protocols recommended by the manufacturer. The sequences were searched for sequence similarity through BLAST (22), and compared to reference sequences in BLAST and downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/).

**Results**

*Adult bioassay*

After a 24-hour recovery period from the 60-min insecticide exposure, the adult bioassay showed that the population of Jazan *Ae. aegypti* mosquitoes was susceptible to cyfluthrin (100%) and resistant to permethrin (84%) and lambda-cyhalothrin (77%) (Table 2).

*AS-PCR of kdr mutations in resistant *Ae. aegypti* mosquito samples*

AS-PCR is considered effective in determining the *kdr* mutations S989P, V1016G and F1534C in individual mosquitoes; these may be responsible for resistance to cyfluthrin, permethrin and lambda-cyhalothrin pyrethroids in Jazan region. Figure 1 and Tables 3 and 4 summarize the presence of the 3 mutations and mutation frequencies (R% = RR% + (0.5 × RS%)).

From all the resistant mosquito samples tested against lambda-cyhalothrin, 1 sample was observed as heterozygous [RS% = RS/(total samples) × 100] for the 3 mutations.

The S989P mutation was not detected [ND% = (ND/(total samples) × 100] in 13 samples (81.2%) resistant to permethrin and 7 samples (39%) resistant to lambda-cyhalothrin. This mutation was detected as homozygous [RR% = (RR/(total samples) × 100] in 3 out of 16 permethrin-resistant samples (with low frequency 18.8%), whereas in 18 lambda-cyhalothrin resistant samples it was detected in 1 sample (RS, 5.5%) and 10 samples (RR, 55.5%) with frequency 58.3%.

The V1016G and F1534C mutations presented in all resistant samples for the 3 pyrethroids used, with high frequency, sometimes reaching 100% as in the samples resistant to cyfluthrin and permethrin, and decreasing to 97.2% in the samples resistant to lambda-cyhalothrin because 1 sample expressed as RS.

*AS-PCR of kdr mutations in susceptible *Ae. aegypti* mosquito samples*

The S989P and F1534C mutations were detected as RS with low frequency, ranging from 2.5–10.0% in the 3 pyrethroid-susceptible samples (Table 5).

Likewise, mutation V1016G was expressed as RS in 55% of the cyfluthrin-susceptible and lambda-cyhalothrin-susceptible samples, and 67% of the permethrin-susceptible samples. It was also detected as RR in 15% of the cyfluthrin-susceptible and 13% of the permethrin-susceptible-samples. The V1016G mutation frequency ranged from 22.5% to 46.5%.
Discussion

In Saudi Arabia, entomological surveys indicated 4 Aedes species; Aedes vittatus, Aedes vexans arabiensis, Aedes aegypti and Aedes caspius (23). Among these, Aedes aegypti is known as the important vector of dengue fever in the Jazan region. The resistance of this species to pyrethroid insecticides has been reported in many countries worldwide, e.g. Grand Cayman, Mexico, China, Thailand, Malaysia, Brazil, Latin America and Indonesia (24).

We found that the susceptibility of adult Ae. aegypti to cyfluthrin was 100%. Similar findings were obtained in a previous study from the Jazan region (15). However, our finding was greater than that previously reported from Mecca (90%) (16). The logical, conventional and frequently repeated explanation that comes to mind this susceptibility to cyfluthrin is that the chemical was recently introduced to the region and the narrow-spread of its use did not provide an opportunity for selective insecticide resistance in Ae. aegypti mosquitoes.

On other hand, this study reflected the high level of resistance that the adult Ae. aegypti population exhibited to lambda-cyhalothrin (susceptibility rate 77%) and permethrin (mortality rate 84%) in the Jazan region. These findings confirm the findings of a previous study carried out in the Jazan region, (15) and were identical to the findings for lambda-cyhalothrin obtained in a study from Mecca (16). The resistance to permethrin agrees with finding obtained from Thailand (25), however, they contrast with the findings of a study from India in which adult Ae. aegypti and Ae. albopictus were found to be susceptible to permethrin (26).

Permethrin and lambda-cyhalothrin resistance could have resulted from the widespread, extensive and successive use of these pyrethroids for more than 10 years in control programmes to limit the population of the vectors in the region. Moreover, the use of pyrethroids to control agricultural pests has also accelerated the development of physiological resistance in these vectors. Likewise, the increased use of low concentration household pesticides aerosols has also accelerated resistance to this chemical (27). It is worth noting that resistance to pyrethroids may also have resulted from the use of DDT in the region as they share the same target in the voltage-gated sodium channel.

The primary target sites of pyrethroids are voltage-gated sodium channels (VGSCs) (28)· and the knockdown resistance mechanism (kdr) and its mutations are highly related to reducing neuronal sensitivity to pyrethroids in several insects, including mosquitoes (29). Detection of these mutations by molecular markers could provide a useful and rapid screening tool for monitoring resistance and helping to target chemical application for vector control (30). The kdr mutations have been reported in Ae. aegypti worldwide, e.g. G923V, L982W, I1011M/V, S989P, V1016G/I, F1534C and D1763Y (31) V1023G, F1565C, I1018M, I1018V, S996P and D1794Y (32).
Three of these mutations have a direct role in pyrethroid resistance, either individually or in combination: V1016G/I (33), S989P (30) and F1534C (7).

In the present study, 3 kdr mutations were detected for the first time in the Ae. aegypti population of Jazan region: F1534C, S989P, and V1016G. We confirmed the presence of the combined mutations (F1534C and V1016G) which are responsible for resistance to permethrin and lambda-cyhalothrin in the adult Ae. aegypti population. It is known that the F1534C kdr mutation in the III6 NaV segment is the most prevalent in the population of Ae. aegypti worldwide and its role in pyrethroids resistance is well defined, either alone or in combination with other kdr mutations (34).

Our results revealed that F1534C and V106G mutations were detected as homozygous (RR) in all female mosquitoes resistant to permethrin with 100% frequency, whereas 94.4% were RR with frequency 97.2% for the 2 mutations in lambda-cyhalothrin-resistant samples. This confirms that F1534C often co-exists with V1016G and this haplotype was strongly associated with permethrin and lambda-cyhalothrin resistance. Similar mutations were detected at high frequency for V1016G, S989P, and F1534C in Mecca and Jeddah (700 km north of Jazan) (2), while in Thailand the V1016G mutation appears to always co-occur with S989P (35).

The combined kdr mutations responsible for deltamethrin resistance in the Ae. aegypti populations in Jeddah and Mecca were V1016G and S989P (2), while in our study resistance to permethrin and lambda-cyhalothrin resulted from combined V1016G and F1534C mutations. This disparity may be related to the discrepancies in the insecticides used to control vector-borne diseases in the 3 areas. Additionally, it is widely accepted that F1534C mutation alone confers permethrin resistance (36). The combined V1016G and F1534C mutations have been shown to increase the resistance to deltamethrin in Ae. aegypti populations of Brazil and Mexico (37,38).

The S989P mutation, on other hand, was detected as RR in 18.8% with low-frequency rate (R) of 18.8% and in 55.5% with (R) 58.3% in permethrin-resistant and lambda-cyhalothrin-resistant samples, respectively. We speculate from the presence of the S989P mutation as RR in 3 permethrin-resistant (out of 16), and undetected in 13 resistant samples that this mutation was negatively correlated to permethrin resistance. This finding agrees with the results of Du et al. (34), who reported that S989P mutation has no effect on permethrin sensitivity on its own or in combination with the V1016G mutation.

Similarly, the V1016G mutation appeared as homozygous (RR) and heterozygous (RS) at high frequency in some samples susceptible to permethrin, cyfluthrin and lambda-cyhalothrin.

The 1534 mutation was never detected as RR, yet observed as heterozygous (RS) with low-frequency in the same samples. Apparently, the effect of the V1016G and S989P mutations on
resistance to permethrin and lambda-cyhalothrin pyrethroids appears to be weaker than that of the F1534c mutation.

**Conclusion**

In this study, cyfluthrin was found to be the only pyrethroid used in Jazan region effective against adult *Ae. aegypti*. The combination of F1534C and V1016G mutations were found most common in the resistance of the adults *Ae. aegypti* population to permethrin and lambda-cyhalothrin. Further studies should be conducted to determine the frequencies and changes among these *kdr* mutations and their role in the resistance to pyrethroids in the region.

The early detection of resistance alleles is considered essential for the successful implementation of insecticide resistance management strategies by providing early warning for insect resistance.

The increasing urbanization and extensive usage of pesticides along with the rising levels of pyrethroid resistance in the Jazan region necessitate the early adoption of proactive monitoring and management programmes for insecticide resistance.

**Funding:** None.

**Competing interests:** None declared.

**References**


Table 1. Specific primers used to amplify sodium channel gene mutations in *Ae. aegypti*, Jazan region of Saudi Arabia, 2017

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Primer</th>
<th>Sequence 5 – 3</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S989P</td>
<td>Outer M1 – For</td>
<td>AATGATATTAACAAAAATTGCGC</td>
<td>594</td>
</tr>
<tr>
<td></td>
<td>Outer M1 – Rev</td>
<td>GCACGCCCTCTAATATTGATGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inner M1 – S</td>
<td>GCGGCGAGTGGATCGAAT</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>Inner M1 – P</td>
<td>GCGGCGAGTGGATCGAAC</td>
<td>240</td>
</tr>
<tr>
<td>V1016G</td>
<td>Outer M2- For</td>
<td>GCCACCGTAGTGATAGGAAATC</td>
<td>592</td>
</tr>
<tr>
<td></td>
<td>Outer M2 – Rev</td>
<td>CGGGTTAAGTTTCGTAGTAGGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inner M1 – V</td>
<td>GTTTCCCACCTCGCACAGGT</td>
<td>348</td>
</tr>
<tr>
<td></td>
<td>Inner M1 – G</td>
<td>GTTTCCCACCTCGCACAGGG</td>
<td>348</td>
</tr>
<tr>
<td>F1534C</td>
<td>Outer M3 – For</td>
<td>GGAGAACTACACGGGAGAAC</td>
<td>517</td>
</tr>
<tr>
<td></td>
<td>Outer M3 – Rev</td>
<td>CGCCACTGAATTGAGATAGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inner M1 – F</td>
<td>GCCTGAAGAACGACCAGCGA</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td>Inner M1 – C</td>
<td>GCCTGAAGAACGACCAGCGA</td>
<td>248</td>
</tr>
</tbody>
</table>
Table 2. Bioassay test for susceptibility to three pyrethroids in adult *Aedes aegypti* mosquitoes, Jazan region of Saudi Arabia, 2017

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>No. mosquitoes tested</th>
<th>Susceptibility (%)</th>
<th>Resistance status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyfluthrin</td>
<td>100</td>
<td>100</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Permethrin</td>
<td>100</td>
<td>84</td>
<td>Resistant</td>
</tr>
<tr>
<td>Lambda-cyhalothrin</td>
<td>100</td>
<td>77</td>
<td>Resistant</td>
</tr>
</tbody>
</table>

Table 3. Bioassay test: distribution of three mutations in resistant *Aedes aegypti* samples of the mosquitoes

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>No. of samples</th>
<th>F1534C</th>
<th>V1016G</th>
<th>S989P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SS</td>
<td>RR</td>
<td>RS</td>
</tr>
<tr>
<td>Cyfluthrin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Permethrin</td>
<td>16</td>
<td>0</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Lambda-cyhalothrin</td>
<td>18</td>
<td>0</td>
<td>17</td>
<td>1</td>
</tr>
</tbody>
</table>

ND = not detected; RR = resistant mutant homozygote; RS = mutant heterozygote; SS = susceptible homozygote.

Table 4. Frequency of the S989P, V1016G and F1534C mutations in resistant mosquitoes for cyfluthrin, permethrin and lambda-cyhalothrin

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>No. of samples</th>
<th>S989P (%)</th>
<th>V1016G (%)</th>
<th>F1534C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SS + ND</td>
<td>RR</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Cyfluthrin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Permethrin</td>
<td>16</td>
<td>81.2</td>
<td>18.8</td>
<td>18.8</td>
</tr>
<tr>
<td>Lambda-cyhalothrin</td>
<td>18</td>
<td>39</td>
<td>55.5</td>
<td>58.3</td>
</tr>
</tbody>
</table>

SS = susceptible homozygote; ND = not detected; RS = mutant heterozygote; RR = resistant mutant homozygote; R = mutation frequencies.
Table 5. Number of positive samples of the three mutations in susceptible samples in the bioassay test

<table>
<thead>
<tr>
<th>Insecticide</th>
<th>No. of samples</th>
<th>S989P</th>
<th>V1016G</th>
<th>F1534C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SS</td>
<td>RS</td>
<td>RR</td>
</tr>
<tr>
<td>Cyfluthrin</td>
<td>20</td>
<td>14</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Permethrin</td>
<td>15</td>
<td>11</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Lambda-Cyhalothrin</td>
<td>20</td>
<td>18</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

| Frequency (%)     |                |       |        |        |        |       |       |        |        |       |       |        |        |
| Cyfluthrin        | 20             | 95    | 5      | 0.0    | 2.5    | 30    | 55    | 15    | 42.5   | 80    | 20    | 0.0   | 0      |
| Permethrin        | 15             | 80    | 20     | 0.0    | 10     | 20    | 67    | 13    | 46.5   | 86.6  | 13.4  | 0.0   | 0      |
| Lambda-Cyhalothrin | 20             | 90    | 10     | 0.0    | 5      | 45    | 55    | 0.0   | 22.5   | 90    | 10    | 0.0   | 0      |

SS = susceptible homozygote. RS = mutant heterozygote. RR = resistant mutant homozygote; ND = not detected.
Figure 1. Agarose gel electrophoresis of S989P, V1016G and F1534C mutation [sequencing of the F1534C mutation revealed that it is in close similarity to some Asian VGSCs, e.g. India (gen bank accession number KM519597.1 and...
KM677280.1), Thailand (gen bank accession number EU792890.1), Japan (gen bank accession number AB909019.1) and the United States of America (gen bank accession number KC107440.1)